P R O G R E S S R E P O R T

ON

FURTHER STUDIES ON ISOLATING AND TESTING AN ANTIMICROBIAL AGENT FROM ACORNS

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FURTHER STUDIES OF ANTIBACTERIAL PROPERTIES OF ACORN EXTRACTS

ABSTRACT

Extracts of acorns from the following species of Quercus - macrocarpa, nigra*, virginiana, and stellata - were tested for antibacterial properties. Extract of Q. nigra had the greatest antibacterial properties against Staphylococcus; therefore, all experiments were conducted using this extract.

Mice tolerated up to 700 milligrams of the crude extract per kilogram body weight when injected intraperitoneally, and up to 10,000 milligrams of the crude extract per kilogram body weight when administered orally and subcutaneously.

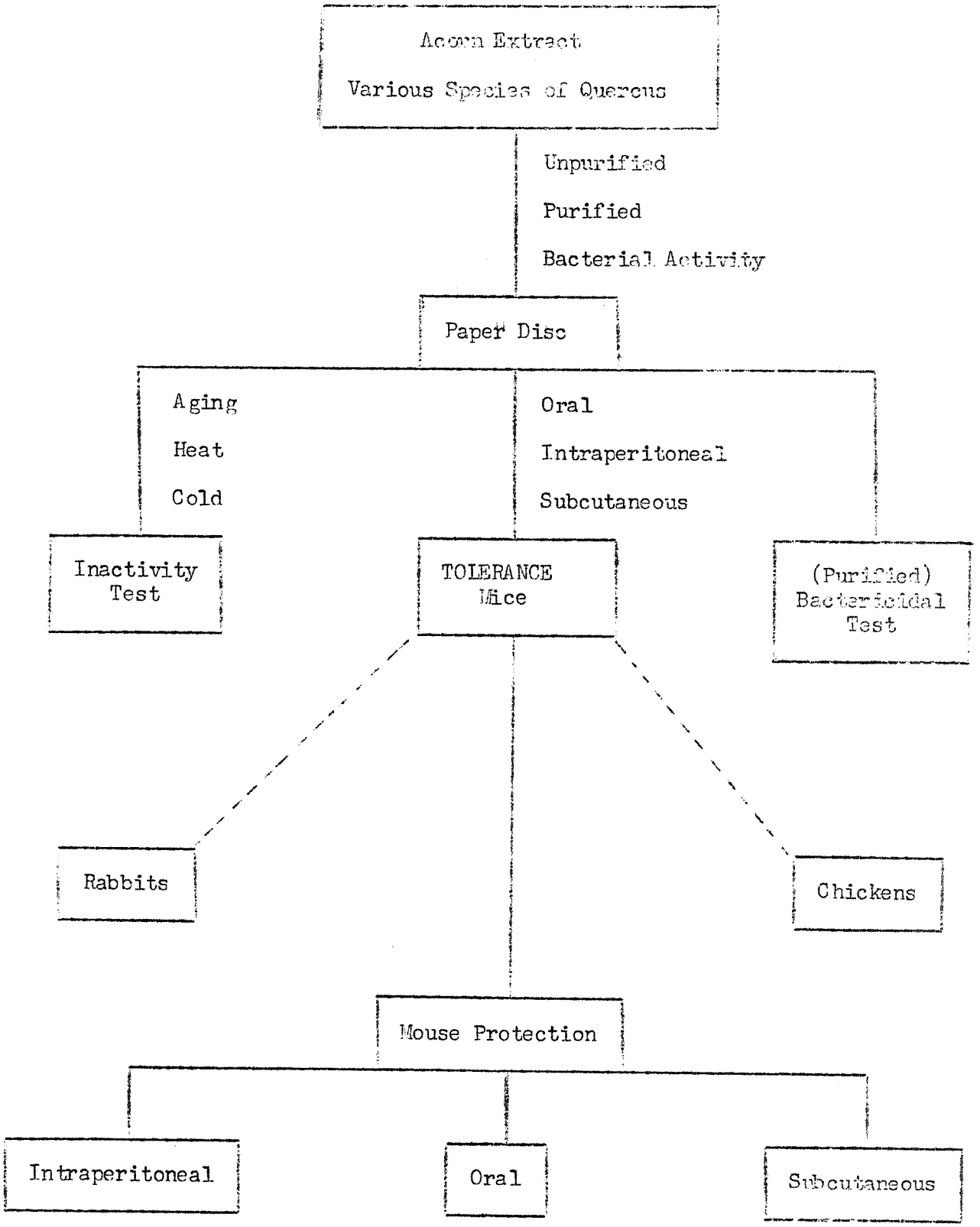
The crude extract was "purified" by passing it through G-75 Sephadex. Mice tolerance to the "purified" yellow fraction of the extract was tested up to 5,000 mg/kg body weight. The extract was administered intraperitoneally, subcutaneously, and orally with all mice surviving. The results from mice protection experiments against Staphylococcus aureus, using the crude and purified extracts, were perplexing in that mice succumbed when the extracts were administered by all routes previously non-toxic, even though in vivo the extracts killed the bacteria below the limits of the extracts administered. Studies are underway to determine why such results were obtained.

Preliminary experiments were conducted in chickens by applying the extract to artificial, topical infections with Staphylococcus. The extract protected the chickens against infections.

Other fractions of the extract collected off the Sephadex column, particularly those that showed antibacterial properties, are being incorporated in a series of investigations.

*The species referred to as <u>cinera</u> in the body of the report has been reclassified as <u>nigra</u>.

FLOW PLAN OF PROCEDURE



Dotted line (-----) Future tests

INTRODUCTION

The presence of curative and antimicrobial substances in plants and their products are widely distributed in the plant kingdom, and knowledge of their existence is generally known. However, search is continued for new and more impressive discoveries for material of medicinal importance. (2,3,4,5,6,7,8) Our study is a part of the array of investigations to contribute additional information to this aspect of science.

In a previous study, Dooley and Gibson (1) reported that an 18% purified acorn extract of Quercus macrocarpa protected mice against a lethal dose of Staphylococcus aureus Smith. The present study is concerned with exploring the possible antibacterial property of extracts of acorns of other species of Quercus. The work also includes administering the extract orally and subcutaneously as well as intraperitoneally. Since the crude (unpurified) as well as the purified extracts are lyophilized, a more specific dosage can be administered.

The procedure differs from that used in the previous study in that the mice are infected before the extract is administered to ascertain its protective ability against inoculated lethal doses of Staphylococcus. Some of the experiments will repeat the procedure of the previous study by administering the extract before inoculating the mice with lethal doses of Staphylococcus. Chemical and physical properties of the different fractions collected off the Sephadex column, as well as their antibacterial properties, will be studied.

Preliminary studies were initiated to determine if protection can be established by local applications of the extract to experimental topical infection.

MATERIALS AND METHODS

The meaty portion of acorns from four species of Quercus (oaks), Q. macrocarpa, Q. virginiana, Q. stellata, and Q. cinera, was blended in distilled, demineralized water to make an 18% solution. The resulting mixture was filtered through Whatman filter paper #1, and the filtrate was paper-disc tested for antibacterial property. This will be referred to as the crude (unpurified) extract. Q. cinera displayed greater bacterial inhibition than any of the species, and it is currently being used in a series of experiments.

A considerable supply of Q. cinera acorns was blended and lyophilized. The lyophilized material was paper-disc tested for antibacterial activity, and the remaining supply was stored in air-tight, amber-colored bottles at 4°C for further use.

Mice tolerance to the crude (unpurified)extract was conducted by administering different concentrations orally, subcutaneously, and intraperitoneally. Female, ICR Swiss strain mice, 18-22 grams, were used in all experiments.

This was followed by conducting mice protection tests with different concentrations of the crude extract through the different routes of administration.

An 18% solution of lyophilized, crude extract was made up in a buffered solution and passed through G-75 Sephadex in a chromatographic column. The following fractions were collected after voiding the buffer in which the Sephadex was prepared: (1) milky white, (2) clear, (3) lemon yellow, (4) yellowish-brown, and (5) purple.

The different fractions were paper-disc tested for antibacterial property, and the remains of each were lyophilized and stored in air-tight, amber-colored bottles at 4°C for further use.

Because of its effectiveness in bringing about inhibition of bacterial growth, the lemon yellow fraction (purified extract) was selected to conduct the first series of tests: (1) Bactericidal test, and (2) Mice tolerance test where the extract was administered orally, subcutaneously, and intraperitoneally.

After establishing mice tolerance, a mouse protection test was conducted by injecting mice with lethal doses of Staphylococcus to determine if administration of the extract by different routes would give the mice protection.

pH of the crude extract and different fractions of the purified material was taken. Since the yellow fraction was on the acid side, it and the crude extract were adjusted to a pH of 7 and retested for bactericidal activity, tolerance by mice, and mouse protection.

An 18% solution of the yellow fraction was dialyzed and adjusted to a pH of 7. It was paper-disc tested for antibacterial property and used in mouse protection experiments.

Preliminary topical infection experiments were conducted using six-week-old White Leghorn chickens. 12 chickens were divided into four groups of three each, and the feathers were plucked from the right thigh. The plucked areas of three groups were cleaned with alcohol and scarified with sandpaper; these areas were then swabbed with an 18-hour culture of Staphylococcus. One group was painted with an 18% solution of purified, yellow extract as soon as the culture was dry; another group was painted 30 minutes after drying, and the third group was not painted with the extract.

The painted groups were repainted daily for six days. All groups were examined daily to check for development of an infection. At the end of the sixth day, blood was drawn to determine coagulation time and white blood cell count.

RESULTS

Unpurified extracts, both lyophilized and unlypphilized, of acorns of all species, showed antibacterial action when paper-disc tested. The extract of Q. cinera showed the greatest amount of inhibition as compared with other species of Quercus. The extracts were more effective against Staphylococcus than against E. coli and B. subtilis. (Table I) Since Q. cinera extract showed the greatest amount of inhibition, all experiments were limited to this extract.

The lyophilized, crude extract was used to test its toxicity on mice, or, we may say, to test the tolerance of mice to the extract. In a previous study, the unpurified extract of Q. macrocarpa was toxic when more than 12 ml of an 18% solution was injected intraperitoneally (1). However, the mice could tolerate dosage of the Q. cinera extract up to 700 mg per kilogram body weight. When the extract was administered orally and subcutaneously, the tolerance was much greater. (Table II)

A culture of Staphylococcus aureus Smith was prepared to conduct mouse protection experiments using the extract administered by different routes. An overnight broth culture was diluted 1:10, 1:100, 1:1,000, and 1:10,000 and injected intraperitoneally into mice. Dilutions of 1:10 and 1:100 killed the mice within a period of 24 hours; mice survived dilutions above the 1:100. (Table III)

Mice were injected intraperitoneally with .5 ml of the 1:10 dilution of bacteria one hour prior to administering the crude extract by the different routes. (Table IV) In all cases, the mice were killed.

Each fraction collected off the Sephadex column was paper-disc tested for bacterial inhibition. The yellow (referred to as the pure fraction) and the

yellowish-brown fraction showed great antibacterial action whereas the other fractions showed no effect. (Table V)

A bactericidal test was run on the 18% purified extract. (Table VI) This fraction showed exceedingly great antibacterial action.

Mouse protection test was conducted with the purified extract using the same procedure followed in using the crude extract. In this case, however, the inoculant was injected 30 minutes prior to administering the extract. (Table VII) In each case - intraperitoneally, subcutaneously, and orally - the mice died.

The purified, yellow extract was injected intraperitoneally up to 5,000 mg/kg body weight to see if the extract were responsible for death. Mice were also injected with the buffer used in making up the Sephadex. In neither case was there any mortality. (Table VIII)

Chickens failed to develop carbuncles and infections where the scarified, bacteria-swabbed area was painted with the 18% extract. The unpainted, bacteria-swabbed areas showed carbuncles indicating the development of an infection.

The white blood cell count of the chickens was normal where the areas were painted with the extract; blood of those not treated showed an elevated white cell count.

Blood from the treated chickens coagulated within the expected time. The blood of untreated chickens failed to coagulate until it had been in the refrigerator for some time.

DISCUSSION

As stated in the abstract, the species of Quercus whose acorns were used exclusively in this study and previously identified as cinera in this report was reclassified as nigra; consequently, this new classification should be substituted whenever cinera is used.

The antibacterial property of the liquid, crude extract lost none of its power when lyophilized. Lyophilizing the extract, however, permitted a more exact preparation of concentrations to be used and was adhered to in making all solutions used throughout the experiments.

18% solutions were made from the freeze dried material. The antibacterial property of the extract was more effective against Staphylococcus than against E. coli and B. subtilis (Table I). The ability of the mice to tolerate large quantities of the crude extract by intraperitoneal injections and by the other routes were gratifying (Table II). Tests with a previous species of acorn had afforded protection for the mice against lethal doses of bacteria, and we hoped that the crude extract would give the same type of reaction.

The reason for experimenting with larger concentrations of the extract was to determine the largest amount of the extract that the mice could tolerate. Rather than to proceed in an orderly, mathematical progression with the extract after reaching the 300 mg/kg body weight tolerance, we started with the higher concentrations and proceeded downward. We found that mice could tolerate 500 mg/kg body weight when the extract was administered intraperitoneally, and up to 7,500 mg/kg body weight when administered by other routes. At the 700 mg/kg body weight, the mice survived; however, there were side effects.

In a previous study where acorns of Q. macrocarpa were used as the source of the extract, mice could not tolerate more than .1 ml of an 18% solution (1).

From this and other studies, it appears that there is a difference in antibacterial properties and toxicity of acorns of different species of Quercus.

With tolerance of the mice to the crude extract established, mouse protection experiments were set up. The mice were injected intraperitoneally with .5 ml of a 10^6 concentration of Staphylococcus aureus, Smith Strain, one hour before administering the extract (Table IV). The strength of the extract administered was greater than the amount it took to kill comparative number of bacteria in vitro. Regardless of the route by which the extract was administered, mortality occurred in each case. From previous experiments we were aware of the presence of the toxic element in the crude extract; however, we eliminated this as the probable cause because the extract in the absence of the inoculum was tolerated. It was also felt that the quantity of the extract should protect the mice against bacterial effect because of its antibacterial action in vitro.

We "purified" the crude extract by passing it through a G-75 Sephadex column. The fractions showing antibacterial property were the yellow (third fraction) and the yellowish-brown (fourth fraction).

From a previous study, the first colored fraction was not only found to be highly antibacterial, but also non-toxic to mice (1). We tested the toxicity of the yellow fraction to mice and found it to be non-toxic; we used this fraction in the mouse protection tests and found that mortality was very high. (Table VII). In this case, however, we administered the extract 30 minutes after injecting a 10^6 concentration of the Smith Strain of Staphylococcus intraperitoneally. Using high dosage of the extract failed to prevent mortality among the mice.

The killing power of the purified extract was observed in the Bactericidal test. Since the amounts administered to the mice were in excess of the amount

needed to kill the bacteria in vitro as seen in the Bactericidal test, it was felt that protection should have been achieved here. The results, however, did not support our anticipation.

Because of results that we obtained in another study, we reversed the procedure by administering the purified yellow extract before injecting the inoculum of bacteria. The dosage ranged from 100 mg/kg body weight to 5,000 mg/kg body weight. The results were the same; however, 5,000 mg/kg body weight of the extract was administered alone without mortality to mice.

A sample of the yellow fraction was adjusted to a pH 7, and a second sample was dialyzed and adjusted to a pH 7; both were used in a mouse protection experiment against Staphylococcus along with appropriate controls. These mice did not die. We found the bacterial inoculum was ineffective; however, this experiment eliminated the possibility that death in the other mouse protection experiments was due to the acid condition of the extract. This was also supported by the fact that in the tolerance experiments where the extracts alone were administered which were on the acid side of pH, there was no mortality among the mice, even when up to 500 mg/kg body weight of the unpurified extract and up to 700 mg/kg body weight of purified extract were administered intraperitoneally, and up to 7,500 mg/kg body weight by other routes.

There is apparently some type of reaction taking place between the bacterial inoculum, the extract, and body tissue that does not occur when the extract is administered alone. It could be that the body tissue is inactivating the extract. It is believed that the extract administered was sufficiently strong to give protection because smaller quantities of the extract were effective in vitro to kill the bacteria.

There are other steps that we hope to pursue to find out why we obtained these unexpected results. We would like to: (1) further purify the extract by

using a different fineness of Sephadex; (2) try the ether fraction of the extract secured from the Sephadex column that was active upon bacteria; (3) use another strain of mice; (4) vary the concentration of the active extracts; (5) use other methods of purifying the active fraction off the Sephadex column; and (6) use a more recent strain of Staphylococcus.

Preliminary experiments testing the ability of the purified extract to prevent a topical infection was encouraging. An 18% concentration of aqueous, purified extract did appear to prevent the development of an infection under the experimental conditions used. The absence of carbuncles and the presence of normal white blood cell counts of treated chicks in contrast to the presence of carbuncles and an elevated white blood cell count in the untreated chicks showed the effectiveness of the extract. The blood of untreated chicks did not coagulate until after refrigeration whereas the blood of treated chicks coagulated at room temperature soon after its withdrawal.

The results indicate that further study is needed, and plans and experiments are being designed to gather additional data.

TABLE I

THE EFFECT OF AN UNPURIFIED EXTRACT

ON DIFFERENT GENERA OF BACTERIA

NAME OF OAK	S. aureus		E. coli		B. subtilis	
	Mix.	F. D.	Mix.	F. D.	Mix.	F. D.
Q. macrocarpa	++	++	+	+	-	-
Q. stellata	++	++	+	+	-	-
Q. virginiana	++	++	+	+	-	-
Q. nigra	+++	+++	+	+	-	-

T A B L E II

TOXICITY TEST OF CRUDE EXTRACT ON MICE

DOSAGE	IP	PO	SUB-Q
10 mg/kg	0/3	0/3	0/3
50 mg/kg	0/3	0/3	0/3
100 mg/kg	0/3	0/3	0/3
300 mg/kg	0/3	0/3	0/3
500 mg/kg	0/3	0/3	0/3
700 mg/kg	0/3	0/3	0/3
900 mg/kg	0/3 (2 dead-32 hrs.)	0/3	0/3
1,100 mg/kg	1/3	0/3	0/3
1,300 mg/kg	1/3	0/3	0/3
2,000 mg/kg	2/3	0/3	0/3
3,000 mg/kg	4/4	0/3	0/3
4,000 mg/kg	4/4	0/3	0/3
5,000 mg/kg	3/3	0/3	0/3
7,500 mg/kg	3/3	0/3	0/3
10,000 mg/kg	2/3	0/3	0/3

IP - Intraperitoneally; PO - Orally; SUB-Q - Subcutaneously

T A B L E III

INOCULANT TEST (24 HRS.) OF STAPHYLOCOCCUS

INOCULANT	DEATH/24 HRS.
Stock	3/3
Dilution 1 (1 - 10)	3/3
Dilution 2 (1 - 100)	3/3
Dilution 3 (1 - 1,000)	0/3

T A B L E IV

MOUSE-PROTECTION TEST

Crude Extract, One Hour After Inoculation

DOSAGE	IP	PO	SUB-Q
200 mg/kg	5/5	-	-
300 mg/kg	5/5	5/5	5/5
500 mg/kg	5/5	4/5	5/5
1,000 mg/kg	-	4/5	5/5
2,000 mg/kg	-	4/5	4/5

IP - Intraperitoneally; PO - Orally; SUB-Q - Subcutaneously

Mice injected with inoculant only showed 5/5 mortality
Mice injected with buffer only showed no mortality

T A B L E V

PH AND BACTERICIDAL EFFECTS OF FRACTIONS

FRACTION	PH	STAPH. AUREUS	E. COLI	B. SUBTILIS
Milky white	7.1	-	-	-
Clear	6.9	-	-	-
Yellow	5.5	++++	-	-
Yellowish-brown	6.95	++++	-	-
Purple	7.1	- - -	-	-

TABLE VI - BACTERICIDAL TEST

Testing Antibacterial Substance in Purified Acorn Extract
(Overnight Culture of Smith Diffuse Diluted Ten-fold)

Inoculum	1	2	3	4	5	10^{-1}	10^{-2}	10^{-3}	10^{-4}	1/2	1/4	1/8	1/16	1/32
Growth-18 Hours	[illegible]	[illegible]	[illegible]	C	568	55	10	3	2	2	2	0	0	0

Tube	Acorn Extract, 18%	BH I	Culture Dil.	Buffer	Growth, 18 Hrs.
1	0.3 ml	0.1 ml	0.1 ml 10^{1} (1)	------	75
2	0.3 ml	0.1 ml	0.1 ml 10^{2} (2)	------	9
3	0.3 ml	0.1 ml	0.1 ml 10^{3} (3)	------	2
4	0.3 ml	0.1 ml	0.1 ml 10^{4} (4)	------	0
5	0.3 ml	0.1 ml	0.1 ml 10^{5} (5)	------	5
6	0.3 ml	0.1 ml	0.1 ml 10^{-1}	------	0
7	0.3 ml	0.1 ml	0.1 ml 10^{-2}	------	0
8	0.3 ml	0.1 ml	0.1 ml 10^{-3}	------	0
9	0.3 ml	0.1 ml	0.1 ml 10^{-4}	------	0
10	0.3 ml	0.1 ml	0.1 ml 1/2	------	0
11	0.3 ml	0.1 ml	0.1 ml 1/4	------	0
12	0.3 ml	0.1 ml	0.1 ml 1/8	------	0
13	0.3 ml	0.1 ml	0.1 ml 1/16	------	0
14	0.3 ml	0.1 ml	0.1 ml 1/32	------	0
A	------	0.1 ml	0.1 ml 10^{-1}	0.3 ml	118
B	------	0.1 ml	0.1 ml 10^{-2}	0.3 ml	726
C	------	0.1 ml	0.1 ml 10^{-3}	0.3 ml	320
D	------	0.1 ml	0.1 ml 10^{-4}	0.3 ml	80
E	------	0.1 ml	0.1 ml 1/2	0.3 ml	15
F	------	0.1 ml	0.1 ml 1/4	0.3 ml	10
G	------	0.1 ml	0.1 ml 1/8	0.3 ml	6
H	------	0.1 ml	0.1 ml 1/16	0.3 ml	3
I	------	0.1 ml	0.1 ml 1/32	0.3 ml	7

T A B L E VII

MOUSE-PROTECTION TEST (24-HR. PERIOD)

Purified Extract, One-half Hour After Inoculation

DOSAGE	IP	PO	SUB-Q
1,250 mg/kg	5/5	5/5	5/5
2,500 mg/kg	5/5	5/5	5/5
5,000 mg/kg	5/5	5/5	5/5

IP - Intraperitoneally; PO - Orally; SUB-Q - Subcutaneously

Mice injected with inoculant showed 4 of 5 were killed
Mice injected with 5,000 mg/kg of extract only showed no mortality
Mice injected with buffer showed no mortality

T A B L E VIII

TOLERANCE OF MICE TO PURIFIED EXTRACT, WATER AND BUFFER

MATERIAL	AMOUNT INJECTED	pH	DEATH
Extract 9% Solution	.5 ml	4.9	0/3
Extract 4.5% Solution	.5 ml	5.0	0/3
Extract 2.25% Solution	.5 ml	5.1	0/3
Demineralized Water	.5 ml	6.3	0/3
Distilled Water	.5 ml	7.0	0/3
Buffer	.5 ml	7.0	0/3

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